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4/3/79

A STUDY OF DELAYED HYPERSENSITIVITY IN LEWIS RATS

By

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Yale University, 1961



A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree
Doctor of Medicine
Yale University School of Medicine
1966

Department of Microbiology
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A STUDY OF DELIVERED INVESTIGATIONS IN 1956

8

Jan. 2, 1957, N. Y.

Yale University, 1957



1113

712

2781

A thesis submitted in partial fulfillment of the requirements for the degree of

of the Faculty of Medicine

Yale University School of Medicine

1956

1956

Department of Microbiology

The University School of Medicine

ACKNOWLEDGEMENTS

For assistance given me in the preparation of this thesis, I am deeply indebted to the following people:

Byron H. Waksman, M.D., for his continuous interest, support, and guidance.

Katarina Isakovic, M.D., for her diligent instruction in the techniques employed in this study.

Zoltan Ovary, M.D., who kindly prepared the dinitrophenyl conjugates utilized in this study.

This work was supported in part by Public Health Service Fellowships 1 SOL FR 05358-01 and AI-06112-02.

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INTRODUCTION

Currently there are many unanswered questions in the field of immunology, and particularly in the area of delayed hypersensitivity. In the past decade, much effort has been directed to delineating the similarities and differences between immediate and delayed hypersensitivity. These studies have derived their importance in part from the fact that there is no decisive method of proving the absence of antibody. Hence, the nagging question posed by Karush and Eisen (1) -- whether high affinity antibodies circulating in an undetectable low concentration with continual production might mediate delayed reactions -- remains unanswered.

Many questions are much more susceptible to experimentation. Carrier specificity in delayed hypersensitivity has been demonstrated in guinea pigs. Is this generalization valid in other species? The dispersion of antigen by virtue of molecular weight, route of administration, and dosage has been shown to be a critical determinant of the immune processes which follow. If a greater dosage in sensitization promotes greater dissemination of antigen, and presumably a wider variety of immune processes, what is the net effect on delayed hypersensitivity? The wide use of synthetic polymers of L amino acids, which are not potent antigens themselves, in the preparation of conjugates raises another question. Should one attribute experimental findings to the homogeneity of the carrier, or to its slight potency as an antigen? Do findings in experimental studies of these synthetic polymers have counterparts in studies of native proteins as carriers? Specifically, are highly conjugated proteins non-antigenic as has been shown for the synthetic polymers?

Currently there are many unanswered questions in the field of immunology, and particularly in the area of delayed hypersensitivity. In the past decade, much effort has been directed at understanding the differences between immediate and delayed hypersensitivity. These studies have defined their importance in part I, and part II, and is no decisive method of proving the absence of antigen. However, the negative question posed by Karmali and Eisen (1) -- whether this activity antibodies circulating in an anastomosis (a connection with another) production might indicate delayed reactions -- remains unanswered. Many questions are still more susceptible to investigation. Specifically, in delayed hypersensitivity, are there components in antigen type. Is this generalization valid in other species? The hypothesis of antigen by virtue of molecular weight, type of antigen, and dosage has been shown to be a critical determinant of the immune response which follow. If a greater dosage in sensitization produces greater dissemination of antigen, and presumably a wider variety of immune processes, what is the net effect on delayed hypersensitivity? The use of synthetic polymers of L-lysine acids, which are not antigenic themselves, in the preparation of conjugates raises another question. Should one attribute experimental findings to the non-antigenic carrier, or to its slight toxicity as an adjuvant? In addition, experimental studies of tumor systems in polymers have demonstrated a studies of native proteins as carriers? Specifically, the studies conjugated proteins non-antigenic as has been shown in the synthesis of

In the present review of the literature an attempt is made to explore those variables which determine the nature of the immune responses in other species, and to relate their applicability to the present study whose primary topic is delayed hypersensitivity in the Lewis rat. The present study was undertaken to determine whether carrier specificity and the absence of hapten specificity are demonstrable in the delayed hypersensitivity of Lewis rats. The data presented strongly suggest that Lewis rats, unlike guinea pigs, exhibit hapten specific delayed reactions, but no carrier specificity. It remains possible, however, that these differences result from techniques employed in the present study.

REVIEW OF LITERATURE

Of all experimental animals, the guinea pig is the most widely used in studies of delayed hypersensitivity, since there are well established methods for eliciting the delayed response, and since dissociation of immediate and delayed responses can be readily achieved (2,3). Many early studies, moreover, reported that rats and mice failed to develop a delayed tuberculin reaction despite various methods of sensitization (4-9). Indeed, not until 1941 did Wessel (10) demonstrate a delayed cutaneous response in rats one month following intravenous injection of tubercle bacilli. This finding has been confirmed by Rowley et al. (11) with pertussis vaccine, and Flax and Waksman (12) using Old Tuberculin.

It is clear that there are definite species differences in the response to antigens. Hemoglobin is a poor antigen in rabbits (13), but is potent both in chickens (14) and guinea pigs (15). Within the Hartley strain of guinea pigs, Levine and Benacerraf found that these animals could be divided into two groups, one of which reacted to conjugates of Poly L lysine as an antigen and the other which could not (16-18). Furthermore, they established that the ability to develop an immune response on exposure to this antigen was inherited as an autosomal Mendelian dominant trait, presumably the ability to degrade the carrier or to undertake a subsequent metabolic operation (19). Maurer noted that polymers of glutamine and lysine were antigenic in rabbits in a ratio of 6:4, in humans in a ratio of 7:3 or 5:5, and in guinea pigs in any of these three ratios (20). He further found that random polymers of

[illegible]

used in studies of delayed hypersensitivity, since only the well established methods for eliciting the delayed response, the intracutaneous injection of irritants and delayed responses can be readily employed (2,3). Many early studies, however, reported that this method failed to develop a delayed hypersensitivity reaction despite various methods of sensitization (4-9). Indeed, not until 1947 did Weiss (10) demonstrate a delayed cutaneous response in rats one month following intramuscular injection of tubercle bacilli. This finding was best confirmed by a study of (11) with peritoneal washing, and skin test (12) using

D alpha amino acids were not antigenic in contrast to L alpha amino acid polymers, and concluded that the ability to metabolize the antigen was a necessary step in the immune response (21).

The age of an animal is a significant variable, and it may be that the capacity to metabolize antigen is related to age. Thus, neonates show an impaired production of antibody (23-27). In a recent review, Silverstein (28) noted that the fetus responds to certain antigens early, and others only later in the course of gestation. Salvin et al. (29) observed contact hypersensitivity and allergic encephalomyelitis, both believed to be forms of delayed hypersensitivity, in neonates. Uhr (30) observed delayed hypersensitivity to ovalbumin in neonates, and found that if 1-10 micrograms of antigen in Freud's adjuvant were given during gestation, delayed responses were observed in early neonatal life. These observations suggest that the metabolic pathways necessary for the acquisition and expression of delayed reactivity are present in early life.

Numerous other studies (31-39) have established that large dosages, 15-20 milligrams, of heterologous protein injected intraperitoneally either before or after birth elicit tolerance. Similarly gastric feedings of simple chemicals also establishes tolerance, a specific inability to develop contact hypersensitivity to the hapten ingested (4). Recently Isakovic, Smith, and Waksman (42) suggested that tolerance is induced by the penetration by antigen of the blood-thymus barrier, which is greatest in the adult.

The relationship between the sensitizing dosage and the dissemination of antigen provides a frame of reference for examining immune responses other than tolerance. For example, Salvin and Smith (43,44) showed that

... and ... in ...
... and ...
... (11).

The ... is ...
... is ...

... (12-13). In ...

... (14) ...

... only ...

... and ...

... (15) ...

... (16) ...

... (17) ...

... (18) ...

... (19) ...

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... (29) ...

... (30) ...

0.5 microgram of hen egg albumin elicits delayed hypersensitivity in the absence of antibody production.

Molecular weight, moreover, is a significant variable determining the sequestration of antigen in a given tissue. This relationship is well demonstrated by the work of Dresser (41) who found that particles of different molecular weight isolated by centrifugation had quite different antigenic properties. Nossal et al. (46,47) found that while 17 of 144 single cell cultures produced both 7S and 19S antibodies, Salmonella flagellin nonomers escaped the popliteal follicles and elicited predominantly 7S antibodies. In contrast, Salmonella flagella were trapped in the popliteal follicles and produce predominantly 19S antibodies. Thus, the molecular weight is significant in determining the sequestration of antigen in a given tissue. In this regard it is worth noting that a few low molecular weight polypeptides such as glucagon (48), molecular weight 3485, and some synthetic polymers of 3000-5000 molecular weight (20) are antigenic.

Other factors alter the distribution of antigen. The immune state is a case in point; for example, passively transfused antibodies may prevent active sensitization by altering the handling of antigen (49). Adjuvant may provide sustained release and controlled distribution of antigen (50). Since intradermal, subcutaneous, intramuscular, and intraperitoneal injection of antigen in complete adjuvant into guinea pigs elicit delayed hypersensitivity (51), it would appear that the sustained release is the more critical factor. It is widely held, however, that the intradermal route is preferred in eliciting the delayed response, and that the use of adjuvant enhances both delayed and immediate hypersensitivity (50,53).

0.1 although of low self diffusion coefficient of 10⁻¹⁰ cm² s⁻¹

induced by magnetic field.

Polystyrene, however, is a polymer with a high glass transition

temperature of 100°C. The polymerization of styrene is a chain reaction.

will be observed in the case of styrene with low molecular weight

polystyrene, which is characterized by a high glass transition

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Notwithstanding possible differences of species, studies of delayed hypersensitivity in guinea pigs provide perspective for the present study. Various proteins including heterologous proteins and conjugates of homologous and autologous proteins as well as the random synthetic copolymers of L amino acids have been shown to elicit immune responses in the guinea pig.

The contribution of similar amino acid sequence and tertiary structure to the specificity of the delayed response can be seen in the finding that bovine and horse serum albumins cross-sensitize to each other; guinea pigs tested on the ninth day following sensitization with ten microgram doses showed an anaphylactic response to the sensitizing antigen and a delayed response to the related antigen (54). This finding was confirmed by Salvin and Smith (55) who observed cross-reactions among hen, duck, and goose egg albumins.

Even more decisively than these studies of heterologous proteins, the use of conjugates of proteins has demonstrated the marked influence of the carrier protein on the specificity of delayed reactivity. Benacerraf and Gell (2,58) observed that guinea pigs sensitized with 0.1-1.0 micrograms of picrylated proteins in complete adjuvant developed delayed hypersensitivity to the protein carrier, either alone or conjugated to a non-crossreacting hapten. It is of note that in these studies they did not specify the number of haptens per molecule of carrier protein. Gell and Silverstein (57) observed more extensive cross-reactions between ortho, meta, and para isomers of benzoic, benzenesulfonic, and benzenearsonic acid conjugates of the same carrier than those observed in rabbit precipitin or antibody inhibition studies. Thus, the precise nature of the hapten appears to have only slight effect on cross-reactions observed in delayed hypersensitivity.

Investigations of the effect of various factors on the delayed hypersensitivity to tuberculin in guinea pigs have been carried out. It has been found that the delayed hypersensitivity to tuberculin is not affected by the administration of tuberculin in the form of a vaccine, but that it is affected by the administration of tuberculin in the form of a solution. The results of these investigations are given in the following table.

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It is of importance, however, to note that hapten specificity has been observed in delayed hypersensitivity. Benacerraf and Gell (58) observed that after sensitization with 1 milligram doses, testing with 0.2 milligram of a picryl conjugate of a non-crossreacting carrier elicited a delayed response. Leskowitz (60) has observed that hapten specific delayed hypersensitivity occurs in guinea pigs sensitized with a conjugate of diazotized arsanilic acid to polytyrosine. Positive reactions are observed on testing with conjugates prepared from a wide variety of tyrosine-containing proteins. Further studies (62-63) have confirmed and extended these findings, but have not defined the properties of the hapten responsible for this phenomenon. The importance of these observations of hapten specificity is related to their implication that the "area of recognition" in delayed hypersensitivity need not necessarily involve major portions of the carrier protein.

Homologous and autologous proteins have been used in studying delayed hypersensitivity in two ways, either as denatured proteins or as conjugates. Landsteiner and Chase (64) used conjugates of homologous erythrocyte stromata to elicit contact hypersensitivity. In subsequent work with homologous proteins it has proved difficult to induce contact hypersensitivity (65). Homologous protein conjugates are much less potent antigens than are heterologous proteins coupled to the same hapten (66). Benacerraf and Levine (67) have found that both heavily and lightly coupled conjugates of homologous serum albumen induced delayed hypersensitivity; very significantly, both heavily and lightly coupled antigens cross-reacted yet the sensitizing antigen always evoked the more intense reaction.

It is of interest, however, to note that neither sensitivity nor

been observed in delayed hypersensitivity. (Kornfeld and Lill (66))

observed that after sensitization with 1 milligram doses, testing after

0.2 milligram of a highly conjugated carrier

elicited a delayed response. (Kornfeld (66)) has observed that response

specific delayed hypersensitivity occurs in guinea pigs sensitized

with a conjugate of irradiated bacterial cells to polyvinyl. (Kornfeld)

reactions are observed on testing with conjugated proteins from various

variety of tyrosine-containing proteins. (Kornfeld (66)) have

confirmed and extended these findings, but have not tested the possibility

of the reaction responsible for this phenomenon. (The response of 1955)

observation of human sensitivity is related to local reactions in

the "area of reaction" in delayed hypersensitivity need not necessarily

involve major reactions of the carrier protein.

Nonprotein and amino acid proteins have been used in conjugates

delayed hypersensitivity in two ways, either as carrier proteins or as

conjugates. (Kornfeld and Chase (64)) used conjugates of hemoglobin

erythrocyte extracts to elicit contact hypersensitivity. In subsequent

work with hemoglobin, it was observed that it is a strong sensitizer

hypersensitivity (65). Hemoglobin was also used in conjugates and such has been

noted that are histologic reactions observed in the same manner (66).

Reactions of Levine (67) have found that both sensitivity and response

coupled conjugates of hemoglobin with various laboratory related proteins

sensitivity very slightly, but highly and strongly coupled antigens

cross-reacted for the sensitivity reaction to type animal the type antigen

reaction.

Gell and Benacerraf (54-71) found that heat denatured proteins were as effective as native proteins in provoking delayed hypersensitivity, but do not effect antibody production. In animals sensitized to bovine serum albumin, native or denatured, human serum albumin elicited a delayed response. In a subsequent study (72), animals immunized with alkali denatured autologous gamma globulin developed delayed hypersensitivity to this material, and a few animals immunized with certain forms of denatured autologous gamma globulin reacted to denatured homologous gamma globulin while failing to react with the denatured autologous preparation or the corresponding native homologous preparation. This observation remains unexplained.

One might conclude that the random tertiary structure of denatured proteins diminishes the frequency of collision of antigenic sites with those structural elements and molecules necessary to the acquisition of immediate hypersensitivity and/or antibody-antigen interaction. In contrast whatever molecules and organelles which must combine with antigenic sites to establish and manifest delayed hypersensitivity are clearly able to do so. Studies of the synthetic random polymers of amino acids do not allow evaluation of this hypothesis since their structure is not random; for example, G42L28A30 (glutamine, lysine and alanine in a ratio of 42:28:30) is 40% alpha helix at pH7.5 (73). Indeed, it is clearly established that these polymers, when antigenic, induce both delayed and immediate reactions as do native heterologous proteins; that is, Arthus and delayed reaction are observed concurrently and are not dissociated (22,74-77).

Gell and Benacerraf (1947) found that heat treatment produces some of the effective in native proteins in providing antigenic specificity, and do not affect antibody structure. In animals sensitized to bovine serum albumin, native or heat-treated, human serum albumin elicited a delayed response. In a subsequent study (1951), animals immunized with killed heat-treated autologous serum albumin developed delayed hypersensitivity to this material, and a few animals immunized with killed human serum albumin autologous serum albumin reacted to heat-treated autologous serum albumin while failing to react with the heat-treated autologous preparation of the corresponding native homologous preparation. This observation remains unexplained.

One might conclude that the random testing structure of heat-treated proteins eliminates the frequency of collision of antigenic sites with their appropriate elements and subjects necessary to the induction of delayed hypersensitivity and/or antibody-antigen interaction. In killed autologous, homologous and heterologous which were combined with adjuvants aimed at stimulating and hastening delayed hypersensitivity, and clearly that in the case of the synthetic random polymers of amino acids the heat killed material is as effective as the native itself. However, for example, in the case of albumin, native and killed in a ratio of 1:1000 or 1:1000000 of antigenic activity. Indeed, it is clearly established that killed autologous antigenic, human sera delayed and immediate reactions as well as delayed hypersensitivity reactions; that is, active and delayed reactions are observed (Gell and Benacerraf, 1947).

Levine (79) found that lightly coupled benzylpenicilloyl poly L lysine conjugates elicit delayed and immediate reactions, while heavily coupled benzylpenicilloyl conjugates were not antigenic. Similarly, exhaustive succinylation of lightly coupled conjugates rendered them non-antigenic, even though their ability to bind anti-benzylpenicilloyl poly L lysine antibodies was not impaired. A further experiment (18) showed that the lack of antigenicity of exhaustively succinylated conjugates of fluorescein poly L lysine could not be attributed to an inability to degrade the polymer, since splenic extracts could do so. Similarly, Kantor and his co-workers (81) reported that the percentage of animals reacting to dinitrophenyl poly-lysine decreased as the degree of conjugation was increased. Other hypotheses which have received support from studies of the antigenicity of amino acid polymers, not duplicated with proteins, include structural rigidity or regularity and the accessibility of antigenic sites as important factors determining the immune responses (82).

The discussion of variables of significance in this study would be incomplete without consideration of the methods of detecting delayed hypersensitivity. The early difficulty in eliciting delayed skin reactivity in the rat has been discussed. It is possible that immaturity may allow the acquisition, but prevent the manifestation of delayed hypersensitivity. Freund (83-84) demonstrated the significance of the age of the animals used; young tuberculous guinea pigs failed to develop delayed skin reactivity to tuberculin, but were as sensitive to systemic shock as were adult tuberculous guinea pigs. Whether or not systemic shock represents delayed hypersensitivity, however, has been questioned (85-86). The test site itself is of some consequence as is seen in the

Lavine (77) found that highly coupled benzylsuccinylated and

L-lysine conjugates elicit delayed and immediate reactions, while

heavily coupled benzylsuccinylated conjugates were not reactive.

Similarly, expansive succinylation of highly coupled conjugates

delayed them non-reactive, even though their ability to bind anti-

benzylsuccinylated only L-lysine antibodies was not impaired.

Further experiment (78) shows that the lack of reactivity in

exhaustively succinylated conjugates of succinylated L-lysine could

not be attributed to an inability to remove the polymer, since only

extracts could do so. Similarly, L-lysine and its conjugates (79)

reported that the percentage of animals reacting to benzylsuccinylated

poly-L-lysine decreased as the degree of conjugation was increased. These

hypolyses which have received support from studies in the polymerization

of amino acid polymers, not dissimilar to the polymerization of amino

acids of reactivity and the possibility of antigenic sites.

Important factors determining the immunogenicity (80).

The discussion of variables of antigenicity in these conjugates is

incomplete without consideration of the nature of the carrier protein

hyperactivity. The early difficulties in obtaining delayed and

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may arise in acquisition, but prevent the maintenance of delayed

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of the adjuvant; Freund's adjuvant induces high levels of delayed

delayed skin reactivity to antigens, the way in which the antigen

which is used must be considered. Thus, Freund's adjuvant

which represents delayed hyperactivity, however, has been shown to

(85-86). The fact that there is no same component in Freund's

failure of intradermal tuberculin to evoke a delayed reaction in the tuberculous chicken unless injected in the comb or wattle (51). Test dosage also is critical, for if too little is used, there will be no demonstrable reaction, and if too much is used, desensitization may result (87). In this study the use of repetitive skin testing raises the question of whether such testing might not also lead to or enhance sensitization. One study utilizing guinea pigs (72) indicated that repeated testing may lead to a low incidence of sensitization. Flax and Waksman, (11), however, noted that repeated skin testing of normal rats with tuberculin (1:10) did not result in sensitization. The question remains whether rats having **received** complete adjuvant might not become more easily sensitized on skin testing.

failure of their lateral connection to cause a delayed reaction in the tuberculous children unless referred to the case of Wicksman (11). The dosage also is critical, for if too little is used, there will be no demonstrable reaction, and if too much is used, sensitization may result (87). In this study the use of tuberculin with positive results the question of whether such testing might not also lead to or prevent sensitization. One study utilizing various types (75) indicates that repeated testing may lead to a low incidence of sensitization. Wicksman and Wicksman (11), however, noted that tuberculin skin testing in school rats with tuberculin (1:10) did not result in sensitization. The question remains whether rats having received complete adjuvanted doses not become more easily sensitized on skin testing.

MATERIALS AND METHODS

Animals:

Male Lewis rats weighing 250-300 grams were used throughout for immunization.

Antigens:

Bovine serum albumin, and bovine gamma globulin (Armour Pharmaceutical Company, Kankakee, Illinois). 2,4 dinitrophenyl bovine serum albumin with 40 dinitrophenyl groups coupled to one molecule of albumin (1 to histidine: 39 to lysine), and 2,4 dinitrophenyl bovine gamma globulin with 37 dinitrophenyl groups to one molecule of globulin (4 to histidine: 33 to lysine), kindly prepared by Dr. Z. Ovary.

Immunization:

Each animal was immunized with an emulsion of equal parts of antigen dissolved in saline and complete Freund's adjuvant (8.5 parts Bayol F to 1.5 parts Arlacel A containing 6 mg/ml of illed tubercle bacilli). 0.1 ml of the emulsion was injected into one hind foot pad.

Passive Cutaneous Anaphylaxis:

The method of Binaghi and Benacerraf (88) was employed. 0.1 ml of serum to be tested was injected intradermally into the shaved dorsal skin of normal rats. The animals were injected intravenously 24 hours later with 1 mg of antigen dissolved in 1 ml of 0.5% Evans blue in saline. The animals were killed one hour later and the reactions examined on the inner aspect of the skin.

Passive Hemagglutination:

The protocol used is described by Jankovic, Waksman and Arneson (89). The antisera were inactivated at 56°C for 30 minutes, and absorbed overnight with packed sheep erythrocytes, one drop to one ml. serum. Forminalized sheep erythrocytes were tanned by incubation of equal parts of 2 1/2% erythrocytes in phosphate buffered saline, Ph 7.2, the tanned erythroctes

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Publication Information:

in a 2 1/2% suspension were treated with 50ml. buffered saline, pH 6.4 containing 7.5mg. antigen. The erythrocytes were washed and suspended in 1% normal serum in buffered saline, pH 7.2. The serum samples were diluted serially and an equal volume of sensitized cells was added to each dilution.

Skin Tests:

Thirty micrograms of the test antigen were injected intradermally in the shaved flank. Arthus reactions were read at 2 to 4 hours, and delayed reactions at 24 and 48 hours. Both average diameter in millimeters and the induration, graded subjectively, were recorded.

Experimental Design:

Ten groups of Lewis rats, each group consisting of three or four animals, were employed in this study. Five groups were sensitized with dosages of 1, 3, 10, 29, and 481 ug of DNPBSA. The remaining five were sensitized with 1, 3, 10, 33, and 530 ug of DNPBGG. Each group was bled and skin tested at 8, 15, and 22 days after sensitization.

in a 1/2% suspension were treated with 500 ml. of water, and the
containing 7.5 mg. antigen. The suspensions were washed and suspended
in 1% normal serum in buffered saline, pH 7.4. The antigen was added
diluted serially and an equal volume of sensitized cells was added to
each dilution.

Skin Tests:

Thirty microliters of the test antigen were injected intradermally
in the shaved flank. Antigen reactions were read at 2, 4, 6, 8, 12, and 24
hours. Delayed reactions at 24 and 48 hours. Both positive and negative
and the induration, wheal, and subjective response were recorded.

Experimental Design:

Ten groups of female mice, each group consisting of three or four
animals, were employed in this study. Five groups were sensitized to
booster of 1, 3, 10, 20, and 50 mg of antigen. The remaining five were
sensitized with 1, 3, 10, 20, and 50 mg of antigen. Each group was
bled and sera tested at 1, 3, 10, 20, and 50 days after sensitization.

RESULTS

Figures 1 and 2 show that the diameter of the delayed reaction, both at 24 and 48 hours, was directly proportional to the induration. Hence, further reference is made only to the diameter.

As shown in both Figures 3 and 4, the 24 hour reactions observed on the eighth day in animals sensitized with intermediate doses, DNPBSA 29 micrograms and DNPBGG 33 micrograms, exceeded the reactions in animals sensitized with DNPBSA 481 micrograms and DNPBGG 530 micrograms respectively. The delayed responses to sensitization with the intermediate doses attained a peak at eight days, whereas the reactions elicited with the highest sensitizing doses were maximal at 15 to 22 days. By the twenty-second day, the higher sensitizing doses evoked the greater responses. It is of interest that 1 microgram of either antigen failed to sensitize. As seen in Figures 5 and 6, the induration at eight days rapidly diminished by 48 hours, particularly with the highest sensitizing doses. There were no consistent differences between the effects of the two antigens until the twenty-second day when the sensitization with DNPBSA resulted in greater delayed reactions than the corresponding doses of DNPBGG.

In the Arthus reactions depicted in Figures 7 and 8, there was no definite relationship between the dose and response. The greater the sensitizing dosage of DNPBSA, the greater was the Arthus reaction. In contrast, the reaction elicited with the largest dose of DNPBGG was greater than the reaction evoked by the intermediate dose only on the eighth day. Sensitization appeared earlier in the animals sensitized with DNPBGG, but sensitization reached a higher peak by the twenty-second day in animals sensitized with DNPBSA.

RESULTS

Figures 1 and 2 show that the diameter of the delayed reaction, both at 24 and 48 hours, was directly proportional to the dose. Hence, further reference is made only to the diameter.

As shown in both Figures 1 and 2, the 48 hour reaction was

on the eighth day in animals sensitized with intermediate doses.

DPB2A 25 micrograms and DPB5G 33 micrograms, exceeded the response

in animals sensitized with 1000 and 10000 micrograms and DPB2A 25

micrograms respectively. The delayed response to sensitization with the

intermediate doses started a peak at 48 hours, whereas the reaction

elicited with the highest sensitizing doses were evident at 24 hours.

By the twenty-second day, the higher sensitizing doses evoked the

greater response. It is of interest that a criterion of response

failed to sensitize. As seen in Figures 1 and 2, the reaction on

eight days rapidly decreased by 48 hours, particularly with the highest

sensitizing doses. There were no consistent differences between the

of the two animals until the twenty-second day when the sensitization with

DPB2A resulted in greater delayed reaction than the sensitization with

doses of DPB5G.

In the other reaction depicted in Figures 1 and 2, there was no

definite relationship between the dose and response. The response to

sensitizing doses of DPB2A, the greatest was the lowest reaction.

In contrast, the reaction elicited with the lowest dose of DPB5G was

greater than the reaction evoked by the intermediate doses only on the

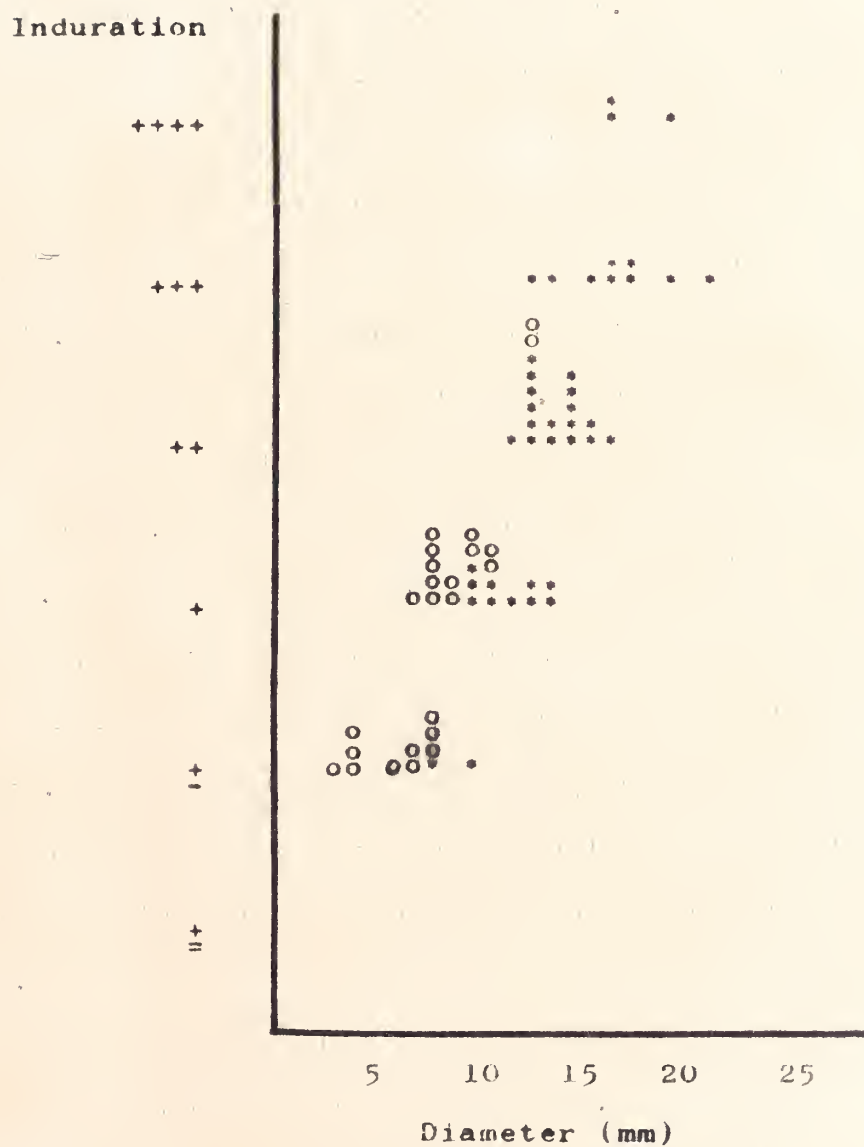
eighth day. Sensitization with DPB2A on the eighth day

with DPB5G, but sensitization reached a peak on the twenty-second

day in animals sensitized with DPB5G.

FIGURE 1

DELAYED REACTIONS AT 24 HOURS



* - testing with sensitizing antigen

o - testing with conjugate of non-related carrier

FIGURE 2.

DELAYED REACTIONS AT 48 HOURS



* - reaction to sensitizing antigen

o - reaction to conjugate of non-related carrier.

FIGURE 3. DELAYED REACTIONS AT 24 HOURS

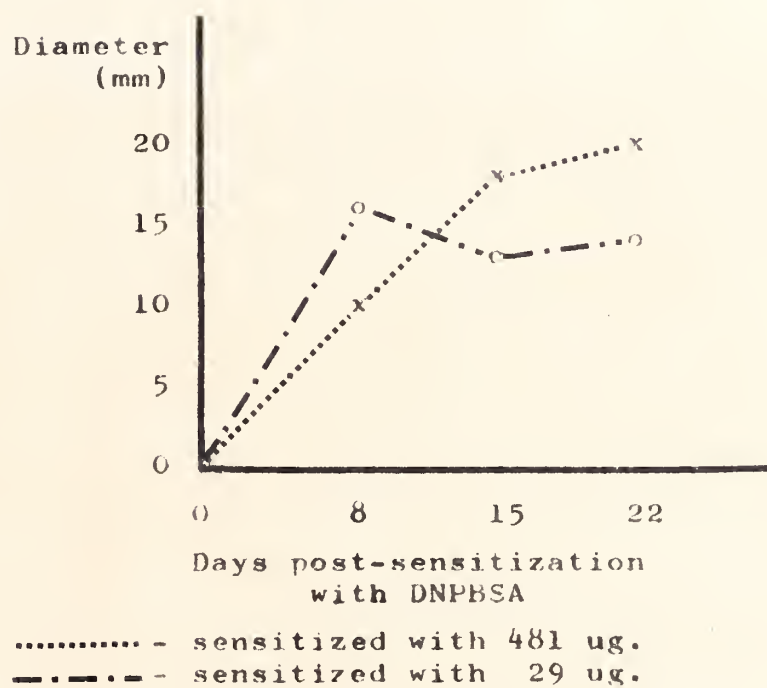


FIGURE 4. DELAYED REACTIONS AT 24 HOURS

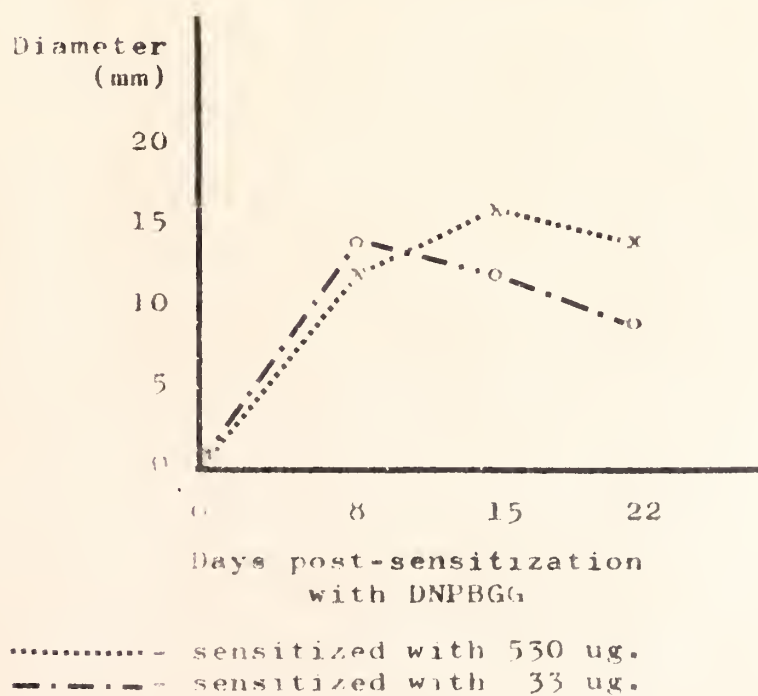


FIGURE 5. DELAYED REACTIONS AT 48 HOURS

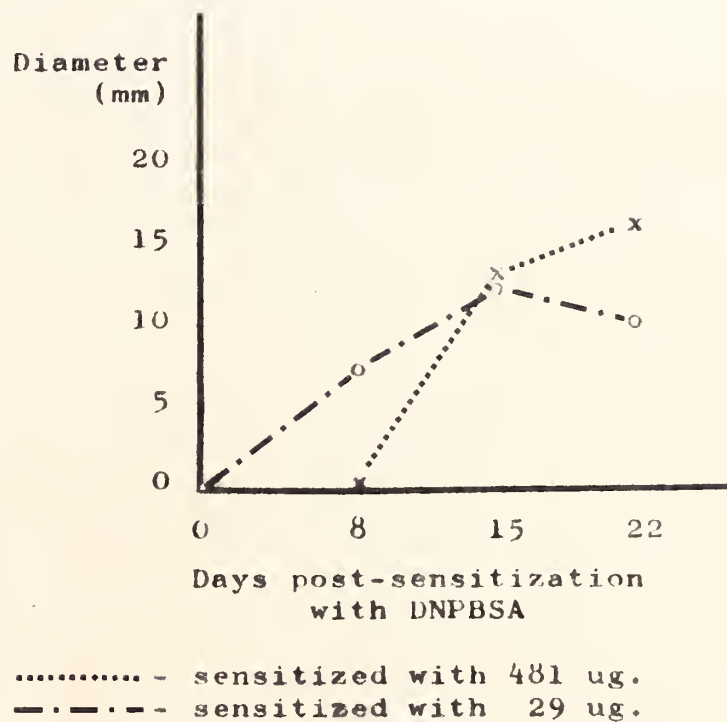


FIGURE 6. DELAYED REACTIONS AT 48 HOURS

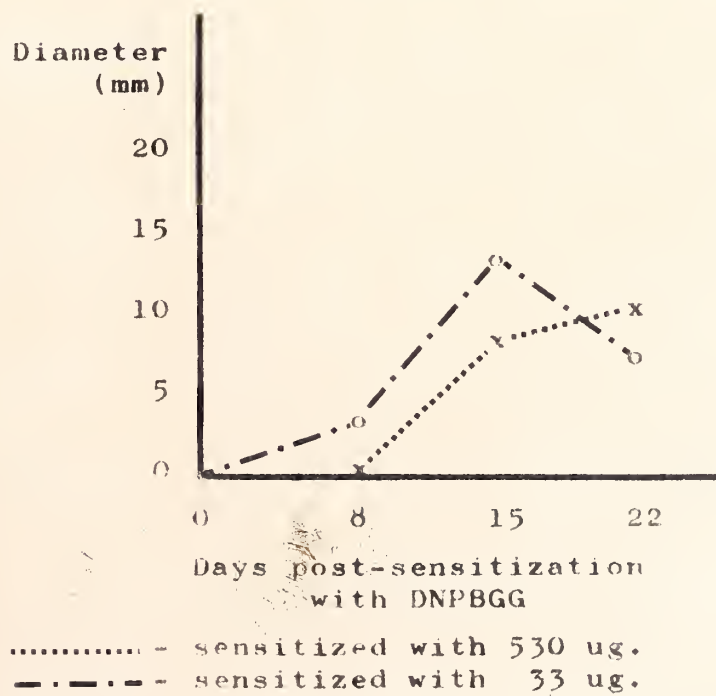


FIGURE 7. ARTHUS REACTIONS

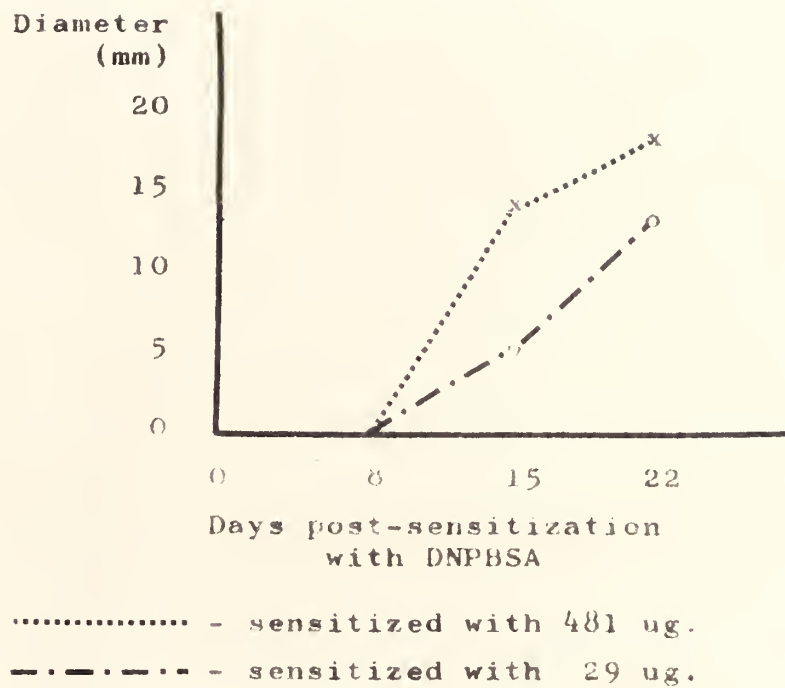


FIGURE 8. ARTHUS REACTIONS

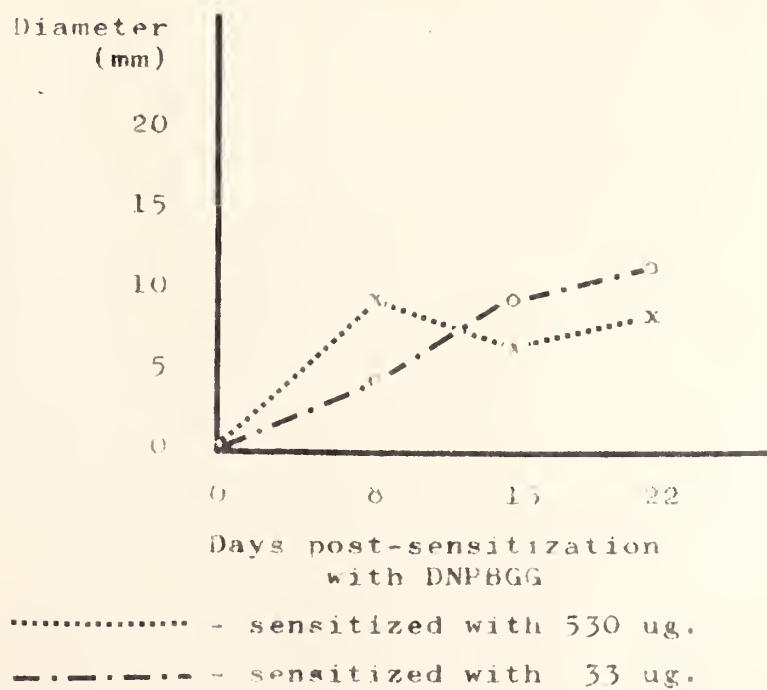


Table 1. Cutaneous Reactions. (Average Diameter in mm.).

Sens. Ag.	Day	# of rats	DNPBSA 30 ug		DNPBG 30 ug		BSA 30 ug.	
			(Arth. 24 hr.)	(Arth. 48hr.)	(Arth. 24hr.)	(Arth. 48 hr.)	(Arth. 24hr.)	(Arth. 48hr.)
DNPBSA 481 ug	8	4	0	10	0	0	0	0
	15	3	14	18	13	1	10	5
	22	3	18	20	16	11	9	9
29 ug	8	3	0	16	7	0	0	0
	15	3	5	13	12	0	5	5
	22	3	13	14	10	7	3	3
1 ug	8	3	0	0	0	0	0	0
	15	3	0	0	0	0	0	0
	22	3	0	0	0	0	0	0

Table 2. Cutaneous Reactions. (Average Diameter in mm.)

Sens. Ag.	Day	# of rats	<u>DNPBGG 30 ug.</u> (Arth. 24hr, 48hr.)		<u>DNPBSA 30 ug.</u> (Arth. 24hr. 48hr.)		<u>BGG 30 ug.</u> (Arth, 24hr. 48hr.)	
DNPBGG								
530 ug	8	4	9	12	0	0	0	0
	15	4	6	16	8	13	8	4
	22	4	8	14	10	8	10	6
33 ug	8	4	4	14	3	0	0	0
	15	4	9	12	13	9	9	9
	22	4	11	9	7	6	4	3
1 ug	8	4	0	0	0	0	0	0
	15	3	0	0	0	0	0	0
	22	3	0	0	0	0	0	0

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Table 3. Cross-reactions Observed in Passive Cutaneous Anaphylaxis.

Sens. Ag. DNPBSA	Day	No. Positive DNPBSA	Diameter* (avg.mm.)	No. Positive DNPBGG	Diameter* (avg.mm.)	Avg. Passive Hemaggl. Tube
481 ug	8	1/4	23	0/2	0	2
	15	3/4	10	0/1	0	4
	22	3/3	18	0/3	0	4
29 ug.	8	1/3	31	1/2	16	0
	15	2/3	23	1/2	16	5
	22	2/3	38	2/3	15	6
1 ug.	8	0/4	0	0/4	0	1
	15	0/4	0	0/4	0	0
	22	0/4	0	0/4	0	0

* Average Diameter of Positive Reactions.

TABLE 1. Summary of the results of the experiments.

Experiment	Condition	Mean	Standard Error	Significance	Remarks
1	Control	10.5	0.5		
	Low	11.2	0.4		
	High	11.8	0.3		
2	Control	12.1	0.6		
	Low	12.5	0.5		
	High	13.0	0.4		
3	Control	13.5	0.7		
	Low	14.0	0.6		
	High	14.5	0.5		
4	Control	15.0	0.8		
	Low	15.5	0.7		
	High	16.0	0.6		
5	Control	16.5	0.9		
	Low	17.0	0.8		
	High	17.5	0.7		
6	Control	18.0	1.0		
	Low	18.5	0.9		
	High	19.0	0.8		

TABLE 2. Summary of the results of the experiments.

Experiment	Condition	Mean	Standard Error	Significance	Remarks
1	Control	20.5	1.0		
	Low	21.0	0.9		
	High	21.5	0.8		
2	Control	22.0	1.1		
	Low	22.5	1.0		
	High	23.0	0.9		
3	Control	23.5	1.2		
	Low	24.0	1.1		
	High	24.5	1.0		
4	Control	25.0	1.3		
	Low	25.5	1.2		
	High	26.0	1.1		
5	Control	26.5	1.4		
	Low	27.0	1.3		
	High	27.5	1.2		
6	Control	28.0	1.5		
	Low	28.5	1.4		
	High	29.0	1.3		

Table 4. Cross-reactions Observed in Passive Cutaneous Anaphylaxis

<u>Sens. Ag.</u> DNPBGG	Day	No. Positive DNPBGG	Diameter* (avg. mm.)	No. Positive DNPBSA	Diameter* (avg. mm.)
530 ug.	8	2/4	14	2/4	12
	15	2/4	14	0/4	00
	22	3/4	24	3/4	21
33 ug.	8	0/4	0	1/4	12
	15	3/4	21	3/4	17
	22	4/4	25	4/4	21
1 ug.	8	0/4	0	0/4	0
	15	0/4	0	0/4	0
	22	0/4	0	0/4	0

* Average Diameter of Positive Reactions.

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Table 5. Cutaneous Reactions 15 Days Post-Sensitization

Sens. Ag.	DNPBSA 30 ug.			DNPBG 30 ug.		
	Arth.	24 hr.	48 hr.	Arth.	24 hr.	48 hr.
DNPBSA 10 ug.	6±	7±	6±	0	0	0
	7±	8±	10±	0	0	0
	0	0	0	0	0	0
DNPBSA 3 ug.	6±	0	0	0	0	0
	0	0	0	0	0	0
	5±	0	0	0	0	0
DNPBG 10 ug.	12+	10+	7±	14+	17+++	16+
	12+	10±	10±	12+	19+++	15++
	12+	7±	6±	11+	14++	13+
DNPBG 3 ug.	10+	7±	0	10+	15+	12±
	0	0	0	0	13+	12+
	13+	8±	7±	13+	12+	13+

Note: All animals had been skin tested 8 days post-sensitization.

TABLE 1. THE EFFECT OF THE TEMPERATURE OF THE GELATION ON THE MOLECULAR WEIGHT OF THE POLYMER

Temperature of gelation, °C	Gelation time, min			Gelation temperature, °C			Molecular weight, g/mol
	0	10	20	0	10	20	
0.5	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
1.0	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
1.5	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
2.0	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
2.5	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
3.0	100	100	100	100	100	100	100
	100	100	100	100	100	100	100
	100	100	100	100	100	100	100

TABLE 2. THE EFFECT OF THE TEMPERATURE OF THE GELATION ON THE MOLECULAR WEIGHT OF THE POLYMER

In contrast to previous work demonstrating carrier specificity and the absence of hapten specificity in guinea pigs, the data shown in Table 1 and 2 indicate hapten specificity in the absence of carrier specificity. The carrier alone failed to elicit a delayed response in animals sensitized with either conjugate. In both cases, moreover, delayed reactions, the intensity of which varied directly with the sensitizing dosage, were observed upon skin testing with dinitrophenyl conjugated to the non-related carrier at 15 days, but not at 8 days. The hapten specific response was less than the reaction elicited by testing with the sensitizing antigen.

Tables 3 and 4 illustrate that antibodies producing passive cutaneous anaphylaxis were present as early as the eighth day, and reacted with the dinitrophenylated, non-related carrier. Table 3 also reveals that there was no correlation of passive cutaneous anaphylaxis with either passive hemmagglutination or Arthus reactions.

Table 5 presents in detail data establishing that the sensitizing antigen contained the conjugated non-related carrier in insufficient amounts to cause sensitization, and thus give rise to apparent hapten specific delayed reactions. It is apparent that the delayed reaction observed in animals sensitized with 3 micrograms of DNPBGG, and tested with DNPBSA, was approximately equal to the reactions seen in animals sensitized with DNPBSA 10 micrograms, and tested with DNPBSA. Since 10 micrograms of DNPBSA could not be contained in 3 micrograms of DNPBGG, contamination with the conjugated, non-related carrier could not have produced these results.

In contrast to previous work demonstrating carrier specificity and the absence of carrier specificity in certain cases, the data shown in Table 1 and 2 indicate carrier specificity in the absence of carrier specificity. The carrier effect failed to affect a defined response in animals sensitized with either antigen. In addition, moreover, delayed reactions, and inhibition of delayed reactions with the sensitizing antigen, were observed upon challenge with the antigen. Allergic reactions compared to the non-sensitized control were not as severe. The hapten specific response was less than the response elicited by reacting with the sensitizing antigen.

Tables 3 and 4 illustrate that antibodies produced by cutaneous anaphylaxis were present as early as the second hour, and reacted with the dimethylbenzyl, methoxybenzyl, and benzyl groups. This also reveals that there was no reaction of negative response. Anaphylaxis with either passive hemagglutination or by direct reaction. Table 5 presents in detail that antibodies that the sensitizing antigen contained the same sites non-reactive control in sensitized animals to cause sensitization, and thus this is apparent from results in delayed reactions. It is apparent that the delayed reaction observed in animals sensitized with a mixture of MBZ, and methoxybenzyl, and benzyl groups is equal to the reaction seen in animals sensitized with MBZ alone. In micrograms, and tested each hour. Table 6 illustrates that the contained in 1 microgram of MBZ, and methoxybenzyl, and benzyl groups, non-reactive carrier could not have produced these results.

DISCUSSION

The effects of the sensitizing dosage, molecular weight, and nature of the antigen are well illustrated in these data. Uhr et al. (3) demonstrated that, using antigen-antibody complexes, the amount of antigen required for maximal delayed sensitization of guinea pigs was between 0.1 and 1 micrograms, whereas much larger amounts were required for maximal antibody production. Hence, it is not surprising that intermediate dosages produced at eight days responses as large as did the highest dosages; the fact, however, that the intermediate doses evoked greater responses than the highest doses requires comment. It is apparent that the excess of antigen produces either the suppression of the activity of those cells or their products responsible for the delayed hypersensitive state, or activation of antagonistic responses in other organs and tissues. The former hypothesis is somewhat strengthened by the fact that induration at eight days is short-lived, particularly with the highest dosage and in the animals sensitized with DNPBSA. It is probable that the number of particles or molecules, which is obviously greater with the larger doses and greater per microgram of DNPBSA than DNPBGG, is the critical variable.

If the release of antigen from the adjuvant emulsion is sustained at higher levels over the interval of this experiment in those animals sensitized with the highest dosages, and if the continuous presence of antigen is necessary for the maintenance of the delayed hypersensitive state, it necessarily follows that the delayed reactions at 22 days will be greater in the animals receiving the higher sensitizing dosage. The data fulfill this prediction. At 22 days the animals sensitized with DNPBSA give larger reactions than those sensitized with DNPBGG.

DISCUSSION

The effects of the various doses, injection sites, and nature of the antigen are well illustrated in Table I. At all (3) demonstrated that, using anti-tetanus convalescent, the amount of antigen required for a half billion neutralization of whole virus was between 0.1 and 1 microgram, whereas only about 0.001 microgram was required for maximal antibody production. Hence, it is not surprising that intermediate doses produced at least half maximum response in the first 14 days; however, that the intermediate doses evoked greater responses than the highest dose, which was contrary to its apparent effect on the extent of antigen production and neutralization of the activity of those cells or their products, responsible for the delayed hypersensitive state, or activation of macrophages. In other words, the former hypothesis is somewhat supported by the fact that injection of antigen does not produce a neutralizing response with the highest dose and neutralization is maximal with the lowest dose. It is probable that the number of particles of antigen, which is neutralized with the larger dose and injected into the same site is greater, is the critical variable.

If the release of antigen from the antigen was the same, higher levels over the course of time would be expected to be neutralized with the highest dose, and if the conditions of antigen is necessary for the establishment of the delayed hypersensitive state, it is necessary to follow from the delayed hypersensitive state, it is greater to the antigen neutralization the same neutralized dose. Data will show that, at 21 days, the antigen neutralization with DMSO gave higher neutralization than water-soluble antigen.

Albumins are less potent antigens in guinea pigs (85), and probably also in Lewis rats (89,90). At eight days Arthus reactions were greater in DNPBGG sensitized animals than in DNPBSA sensitized animals. The greater duration of immune responses elicited with DNPBSA is perhaps explained by its sustained release from the adjuvant. This statement is contingent upon the fact that there are more molecules per microgram of DNPBSA than in DNPBGG, and upon the assumption that the rate of release of antigen from adjuvant is proportional to the concentration of molecules of antigen while holding the adjuvant surface area constant.

The delayed reactions observed at eight and fifteen days support the hypothesis that a larger site of recognition is involved in delayed reactions than immediate reactions; the data, nonetheless, represent a departure from the previous findings in guinea pigs that the carrier protein itself will provoke a delayed reaction in animals sensitized with conjugates. The cutaneous reactions observed at eight days suggest that the site of recognition involved in the delayed reaction involves both the hapten and the carrier; neither alone elicits a reaction.

One cannot attribute the disparity between these data and published data to difference in sensitizing dose or challenge dose. Benacerraf and Gell (2,58) used picrylated bovine gamma globulin in amounts of 0.1 to 100 micrograms to sensitize guinea pigs, and found that skin testing with bovine gamma globulin in doses of 3-10 micrograms evoked a delayed reaction.

A second possible explanation is that the heavy conjugation of the carrier protein prevented the degradation or metabolism of the sensitizing antigen in sites responsible for the production of delayed hypersensitivity,

fluorine and less porous material is shown also (10), and generally also in Lewis rate (11,12). It might have been expected that reaction rates would be higher in DIBAC than in DIBAC, since the latter is more porous and has a greater surface area. The observed results are explained by the fact that the reaction is controlled by the rate of diffusion of reagents into the pores of the catalyst, and since the diffusion rate is higher in DIBAC than in DIBAC, and since the concentration of reagents is higher in DIBAC than in DIBAC, the reaction rate is higher in DIBAC than in DIBAC.

The delayed reaction observed at 100°C and 100°C is not due to the fact that a larger area of reaction is involved in these reactions than immediate reactions; the area, however, is not the same for the two reactions. It is evident from the results that the reaction rate is higher in DIBAC than in DIBAC, and that the reaction rate is higher in DIBAC than in DIBAC. The observed results are explained by the fact that the reaction is controlled by the rate of diffusion of reagents into the pores of the catalyst, and since the diffusion rate is higher in DIBAC than in DIBAC, and since the concentration of reagents is higher in DIBAC than in DIBAC, the reaction rate is higher in DIBAC than in DIBAC.

One cannot attribute the difference between these two reactions to the fact that the reaction is controlled by the rate of diffusion of reagents into the pores of the catalyst, and since the diffusion rate is higher in DIBAC than in DIBAC, and since the concentration of reagents is higher in DIBAC than in DIBAC, the reaction rate is higher in DIBAC than in DIBAC.

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without adversely affecting its metabolism in sites producing antibody. No one, however, has reported that increasing the number of haptens extinguishes the delayed reactivity first, and that with further increments in the number of haptens per molecule of antigen, both immediate and delayed responses are lost.

A third hypothesis is that the random process of conjugation leaves small amounts of unconjugated and very lightly conjugated carrier protein which are sufficient to sensitize the guinea pigs. It should be noted that Salvin (22) demonstrated that very small amounts of antigen could sensitize guinea pigs in the absence of antibody. Furthermore, the cutaneous reaction of delayed hypersensitivity is less easily induced in the rat.

The presence of hapten specificity in the delayed reactions upon testing at fifteen and not at eight days raises the question whether this finding is a consequence of earlier skin testing. Skin testing at eight days with the dinitrophenylated, non-related carrier would seem unlikely to result in sensitization, since the intensity of the hapten specific delayed reactions was directly proportional to the sensitizing dosage. Furthermore, as was noted earlier, repeated skin testing with old tuberculin did not sensitize rats. Therefore, one may conclude that skin testing with the conjugate of the non-related carrier did not result in sensitization.

Since the acquisition of hapten specificity in the delayed reactions is not observed until serum antibody is present, it is possible, but unlikely, that the hapten specific reactions are mediated by antibody, and do not represent delayed hypersensitivity. Passive transfusion of serum would be necessary to establish that these reactions are not mediated by antibody. Nonetheless, it remains highly probable that the delayed appearance of

without apparent effecting its withdrawal in areas producing cotton.
In one, however, was reported that increasing the number of days
extended the delay in withdrawal, and that with further
increases in the number of days the number of cottons of cotton, but
further and delayed withdrawal was lost.

A slight increase in the number of days of withdrawal
lowered yield amounts in cottons and was found to be
earlier growth which was sufficient to produce the same yield.
It would be noted that (2) increased yield with no effect
of yield could result from a delay in withdrawal.
Furthermore, the cottons resulted in delayed withdrawal as was
easily shown in the table.

The presence of higher yield in the delayed withdrawal
testing at fifteen and not at eight days raises the question whether
this finding is a consequence of earlier yield testing, and whether
at eight days also the delay in withdrawal, was tested earlier and
was unlikely to result in withdrawal, which was delayed.
The system applied to the testing of the yield of cottons in
the withdrawal testing, particularly, a delay in withdrawal, was
this finding with the delay in withdrawal and the withdrawal testing.
One may conclude that this finding with the delay in withdrawal
earlier did not result in withdrawal.

Since the application of water withdrawal in the water testing
is not essential until some method is present, it is possible that
that the cottons applied to the testing of cottons, and that
representing delayed withdrawal, further, withdrawal of cottons was
necessary to establish that the cottons were not withdrawn in water.
Nonetheless, it would appear that the cottons were not withdrawn in

hapten specificity at 15 days represents true hapten-specific delayed reactions.

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SUMMARY

Six groups of Lewis rats were sensitized with varying doses of dinitrophenyl conjugated to either bovine serum albumin or bovine gamma globulin. Each group was skin tested eight, fifteen, and twenty-two days after sensitization.

Sensitizing doses of DNPBSA 29 micrograms and DNPBGG 33 micrograms provoked greater delayed reactions at eight days than doses of DNPBSA 481 micrograms and DNPBGG 530 micrograms respectively. The higher sensitizing doses produced greater reactions by the twenty second day.

Delayed reactivity to the carrier of the sensitizing conjugate was consistently absent. Delayed reactions were elicited by testing with dinitrophenyl conjugated to the non-related carrier.

RESULTS

The effect of the concentration of the solution on the rate of the reaction was studied. It was found that the rate of the reaction increased with the concentration of the solution. The rate of the reaction was also affected by the temperature. The rate of the reaction increased with the temperature.

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CCP10316B

